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Review

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Association of Chemotherapeutic Drugs with Dendrimer Nanocarriers: An Assessment of the Merits of Covalent Conjugation Compared to Noncovalent Encapsulation

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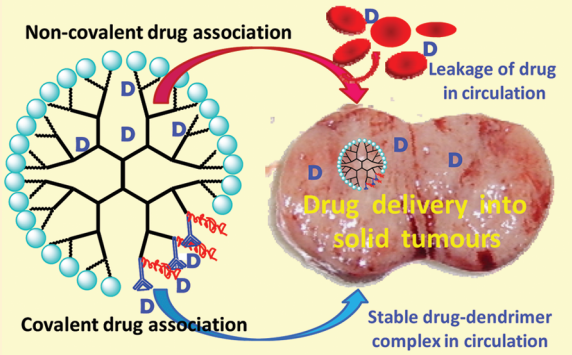
ABSTRACT: Cancer is a leading cause of death within developed nations, and part of this morbidity is due to difficulties associated with its treatment. Currently, anticancer therapy relies heavily upon the administration of small molecule cytotoxic drugs that attack both cancerous and noncancerous cells due to limited selectivity of the drugs and widespread distribution of the cytotoxic molecules throughout the body. The antitumor efficacy and systemic toxicity of existing chemotherapeutic drugs can, however, be improved by employing formulation and particle engineering approaches. Thus, drug delivery systems can be developed that more specifically target tumor tissue using both passive (such as the enhanced permeation and retention effect) and active (through the use of cancer targeting ligands) modalities. Dendrimers are one such system that can be developed with high structural monodispersity, long plasma circulation times and precise control over surface structure and biodistribution properties. Chemotherapeutic drugs can be associated with dendrimers via covalent conjugation to the surface, or via encapsulation of drugs within the structure. Each of these approaches has demonstrated therapeutic benefit relative to the administration of free drug. Thus far, however, there has not been a systematic review toward which drug association approach will provide the best outcomes in terms of antitumor efficacy and systemic toxicity. Hence, the current literature is reviewed here and recommendations are proposed as to the suggested approach to develop dendrimers as tumor targeted drug-delivery vectors.



KEYWORDS: *dendrimer, tumor, drug delivery, drug encapsulation, covalent conjugation*

1. INTRODUCTION

There is much current research into improving chemotherapy using approaches based on nanotechnology and polymer chemistry. In this review, the issues around approaches to improving chemotherapy using such constructs are briefly introduced, before focusing on an interesting class of globular polymers (dendrimers) as nanocarriers for chemotherapy drugs. The nature of association of the drug to the dendritic carrier is critical to the delivery of chemotherapeutics. So far, however, an examination of which of these two approaches is most ideally suited to the development of tumor delivery systems for chemotherapeutic drugs has been limited to a few isolated studies. Consequently, in this review we compare the known outcomes of the two broad drug association strategies to enable a rational conclusion to be drawn about the most likely successful strategies to employ in designing and developing dendrimer-based drug delivery systems.

2. DRUG DELIVERY TO SOLID TUMORS

Chemotherapeutics are traditionally administered as slow intravenous infusions of free drug. However therapeutic efficacy and systemic toxicity may be improved by the use of tumor targeted drug delivery systems. The poor physicochemical properties, such as low aqueous solubility, and/or nonspecific distribution of small molecule chemotherapeutics to healthy tissues limit the effective dose delivered to tumor tissues. Approximately 40% of new chemical entities, including chemotherapeutic drugs discovered through rational design approaches, exhibit poor water solubility, which limits the practical dose that may be administered as a simple solution formulation. Many chemotherapeutics have therefore been formulated using approaches such as complexation, cosolvency and micellar solubilization. Each of these approaches, however, has limitations. The toxicity of the excipients (cosolvents, surfactants, cyclodextrins for example) is a major issue, as is the potential for drug precipitation on dilution in iv fluids for cosolvent and micellar systems. Some steps toward resolving these issues have been made with the recent formulation of

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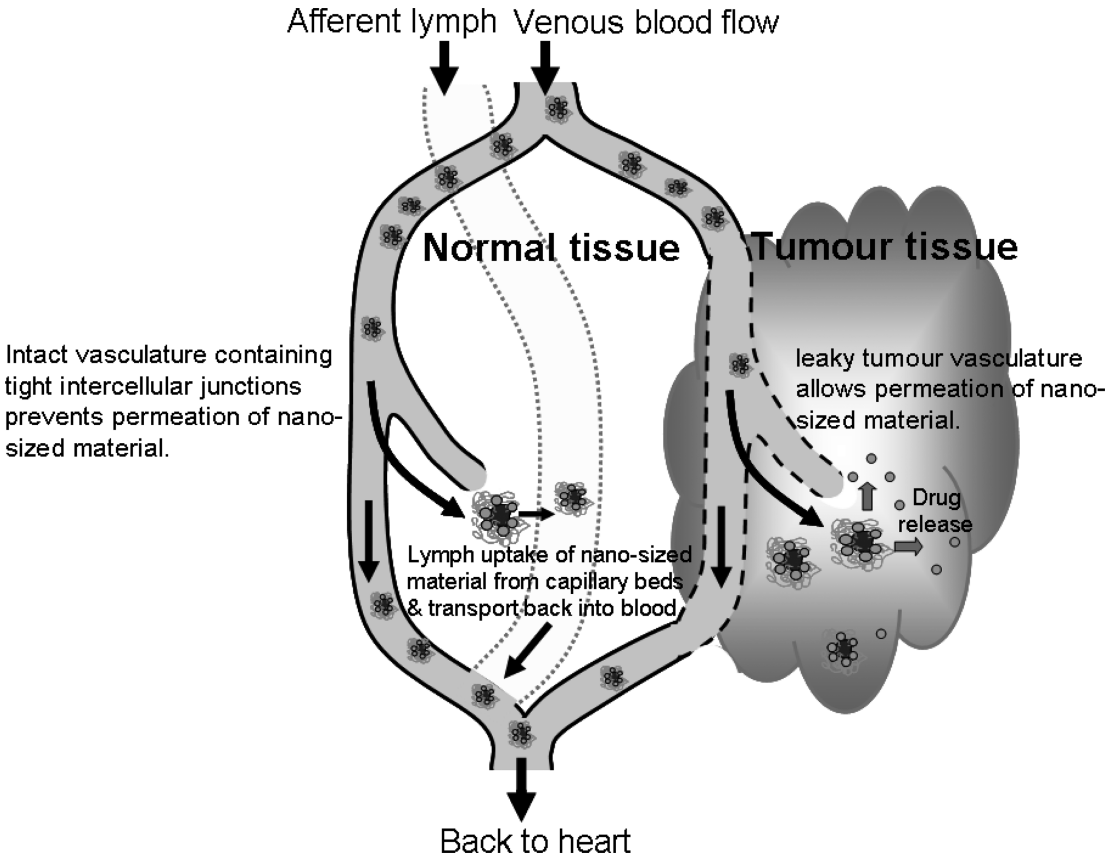

Figure 1. Diagram of the lymphatic and vascular supply to normal tissue and solid tumors. Nanosized particles can permeate into solid tumors due to enhanced vascular permeation and increased retention due to the lack of a functioning lymphatic system, where associated drug molecules are released. The vasculature in noncancerous tissue is generally poorly permeable to circulating particles and macromolecules, although macromolecules such as albumin do extravasate via fenestrated capillary beds or by active transcytosis mechanisms. Once present in the interstitium, they are delivered back to the systemic circulation via the lymphatic system.

solid drug nanoparticles of paclitaxel,[1] however the success of this approach depends critically on the physicochemical properties and protein binding of the drug, and relatively few drugs are likely to be amenable to such formulation approaches. Thus, delivery systems that overcome these major issues are increasingly being sought as means to improve the delivery of chemotherapy drugs to tumors.

2.1. Physiology and Tumor-Targeted Delivery via the Enhanced Permeation and Retention Effect. Solid tumors possess a number of distinguishing features when compared to healthy tissue that can be exploited in drug delivery to improve the tumor-specific distribution of chemotherapeutic drugs. These include the formation of a vascular network with poorly constructed and highly leaky architecture, lack of a functioning lymphatic supply, decreased extracellular pH and the increased expression of various antigens, receptors and enzymes by the tumor cells.[2−5] Many tumors overexpress receptors that can enable more specific targeting of drug carriers by the surface presentation of targeting antibodies and ligands such as Herceptin[6] (which targets HER-2 expressing breast cancer cells), cetuximab[7] (which targets the epidermal growth factor receptor) and folic acid[8] (which binds folate receptors overexpressed by many cancers). However, targeting ligands on carrier systems must be used with care as they may also enable the uptake of cytotoxic drugs in other organs that similarly express high levels of the receptor (for example, the overexpression of folate receptors in the placenta and thyroid), or where receptor binding produces agonist effects that may promote tumor growth (such as the binding of epidermal growth factor to its receptor).[9]

The tightly packed endothelium in the well-formed vasculature in normal tissue prevents the extravasation of nanosized material such as proteins, foreign antigens and colloids. Tumor vasculature, however, possesses loosely packed endothelia with compromised intercellular junctions and a poorly constructed basement membrane that allows the permeation of large material that would otherwise not normally escape blood vasculature.[2,3] Normal tissue also contains a network of lymphatic capillaries that function alongside the vascular supply, which are responsible for maintaining fluid homeostasis and the removal of foreign antigens and other nanosized material from the interstitium.[3] Although lymphangiogenesis takes place in some tumors, the functionality of newly formed lymphatic capillaries in maintaining fluid homeostasis or removing nanoparticulate material has not yet been demonstrated.[10,11] The combination of a leaky vascular network and the absence of a functioning lymphatic system enables macromolecules and particles of an appropriate size to extravasate and to concentrate within solid tumors (Figure 1). This process is known as the enhanced permeation and retention (EPR) effect and is exploited as a means of passive delivery of nanosized material containing chemotherapeutic drugs more specifically to solid tumors.[12,13] The effectiveness of this approach, however, is dependent on the long circulating behavior of the drug carrier since rapid uptake into organs, for

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instance, of the reticuloendothelial system will result in poorer control over systemic toxicity and side effects.

2.2. The EPR Effect and Drug Delivery Systems. The accumulation of drug delivery systems with sufficiently small dimensions on the nanoscale (including nanoparticles, colloids and polymers) specifically in tumors via the EPR effect has been widely investigated. Thus far only liposomal formulations of doxorubicin (Doxil/Caelyx and Myocet) and daunorubicin (DaunoXome) have been approved for clinical use in cancer therapy. Doxil is a PEGylated liposomal formulation with particle size <100 nm in diameter and is used mainly in the treatment of advanced breast and ovarian cancers. Myocet and DaunoXome, however, are unPEGylated liposomal formulations of drug that have different pharmacokinetic properties and as such are indicated for different cancers. The liposomes have diameters in the range of 35−100 nm and as such avoid renal clearance, while also evading uptake by the reticuloendothelial system (RES). The resulting extended residence time in blood enables tumor deposition via the EPR effect.

Liposomes do have a number of drawbacks, however. First, they are known to release drug along a concentration gradient, leading to drug release in plasma and nontarget tissues, although this can be controlled somewhat by modifying the lipid composition.[14−16] Doxil, for instance, has an *in vitro* doxorubicin leakage half-life of 5 days.[17] Liposomes have also been associated with hypersensitivity reactions.[18] Finally, liposome dispersions are statistical mixtures of particles of differing size and composition, and hence from a regulatory and manufacturing standpoint are not as well-defined as small molecule drugs. The potential drawbacks of liposomes have consequently led to the more recent investigation of alternative means of delivering drug selectively to target tissues.

2.3. Tumor-Specific Drug Liberation from Nanosized Drug Delivery Systems. There are often differences in the microenvironment and expression of various enzymes between tumors and normal tissue. These differences can be exploited to facilitate drug release from delivery systems selectively in tumor tissue, as described further in the following sections. In addition, external triggers for drug release may also be employed, relying upon the tumor selective distribution of the delivery system followed by the local application of, for instance, heat or ultrasound to stimulate drug release. In the context of this review, however, biological triggers for drug release are more relevant than external triggers. External triggers have been previously reviewed.[19]

2.3.1. Low Extracellular pH. The extracellular pH of solid tumors is generally lower than that of normal tissue on account of the high rate of glycolysis and inefficient removal of acidic waste products.[4] The extracellular pH can range between 7.8 and 5.7 and is believed to contribute to tumor invasiveness by stripping the extracellular matrix and destroying neighboring noncancerous cells.[20] The lower pH can be used to trigger the release of chemotherapeutic drug from the dendrimer carrier specifically within solid tumors. Release is facilitated either by partition of noncovalently encapsulated drug out of the dendrimer down a pH gradient or by cleavage of an acid-labile functionality covalently linking drug and dendrimer. Specific acid-labile linkers will be discussed in more detail in section 4.2.

2.3.2. Overexpression of Receptors and Enzymes. The overexpression of various enzymes such as matrix metalloproteinases,[21,22] cathepsins,[23] phospholipases[24] and elastases[25] by cancer cells can also be utilized to enable enzyme-mediated release of drug once exposed to the tumor. It should be pointed out, however, that drug targeting and delivery approaches that rely on reduced extracellular pH and the overexpression of enzymes possess drawbacks due to the great heterogeneity in these properties between solid tumor types.[19,26,27] For example, not all tumors overexpress matrix metalloproteinases, and those that do overexpress these enzymes express them to differing degrees. Similarly, the extracellular pH within tumors can vary significantly both between different tumors and within the tumor itself.[20] Hence, it is necessary to understand the biochemical nature of a target tumor prior to beginning treatment with such nanomedicines in order to achieve the optimal deposition of liberated drug in tumor tissue and to minimize exposure of noncancerous tissues to cytotoxic drug.

Nevertheless, it is apparent that nanosized EPR-enabled carriers with potential for sufficient drug incorporation or covalent attachment, and selective drug release in tumor environments have potential to provide improved chemotherapeutic outcomes compared to the use of small molecule chemotherapeutics alone. With this emerging concept in mind, dendrimers have received much attention as an alternative delivery system to liposomes for delivery of chemotherapeutics. Consequently their potential application in the treatment of solid tumors is the focus of this review. A brief introduction of dendrimers and their biopharmaceutical behavior is provided for the unfamiliar reader, before focusing on the mode of drug association with dendrimers and its impact on effective drug delivery outcomes.

3. DENDRIMERS

3.1. What Are dendrimers? Dendrimers are geometric polymeric structures that are prepared through the stepwise addition of multifunctional monomeric units around a central core to form a "treelike" structure (Figure 2). The core may be composed of any functional monomer provided it has at least two functional groups to enable the attachment of additional layers or "generations" that form the bulk of the dendrimer, providing immense versatility in potential structure. The

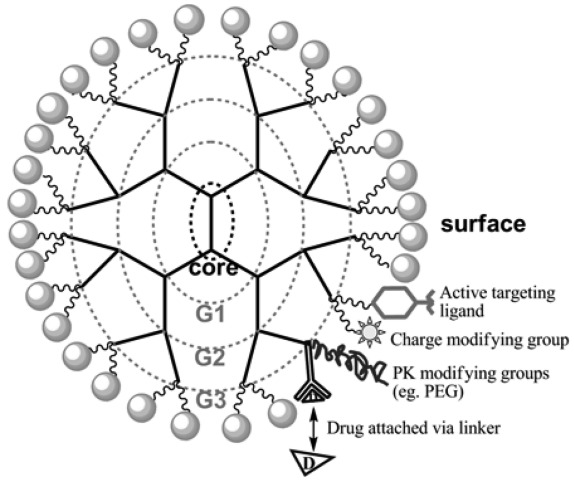

Figure 2. Representation of the layers or "generations" (G) of a dendrimer. The surface may be composed of any appropriate functional group. Symbols representing groups for active targeting, modification of pharmacokinetics and covalently conjugated drug are illustrated schematically.

dendritic structure can be assembled via either a divergent synthesis (involving the stepwise attachment of repeating layers around a central core) or a convergent synthesis (involving mergence of individual segments of the dendrimer, termed "dendrons", in the final step). The dendritic scaffold often contains secondary or tertiary amines that enable the entrapment of drug molecules via hydrogen bonding, ionic binding or hydrophobic interactions. The outer layer possesses a defined number of reactive functional groups that may be modified to influence the valency, solubility, tissue binding, pharmacokinetics or biodistribution properties of the dendrimer. In addition, the surface reactive groups may also be used to covalently attach drug molecules via labile chemical linkages. Using these approaches, dendrimers can be constructed with precise control over the number of generations and surface functionalities, producing structures with typically high monodispersity when compared to the synthesis of traditional polymers which typically produce statistical mixtures of products.

The most commonly investigated dendritic structures are the highly stable polyamidoamine (PAMAM) and polypropyleneimine (PPI) dendrimers. While alternative structures with improved biological lability have been prepared using a polyester or polypeptide scaffold,[28−30] PAMAM and PPI dendrimers are more commonly investigated. This is most likely because they are more readily commercially available and have been commercially available for a longer period of time than other dendrimers. In addition, they have peripheral amine groups that are readily modified. Hence, they are not typically selected for study based on desirable biological properties but more based on their utility in research laboratories and availability. However, large nondegradable dendrimers may not readily undergo urinary elimination, resulting in accumulation in the liver.[31] In contrast, biodegradable dendrimers have an advantage over the more biologically stable dendrimers because the scaffold can degrade into smaller, more readily eliminated fragments.[28] For example, polylysine dendrimers undergo degradation in the body resulting in the liberation of monomeric lysine that may then be reused in protein synthetic pathways.[28]

3.2. *In Vivo* Pharmacokinetics and Biodistribution of Dendrimers. The outer surface groups of unconjugated dendrimers are typically primary amine groups that provide a polycationic charge. These cationic charges can form strong ionic bonds with anionic charges on tissue membranes and cells. On account of this strong tissue binding capability, unconjugated dendrimers often show increased cytotoxicity in *in vitro* assays and can disrupt the tissue membrane, creating pores through which dendrimer may be absorbed.[32,33] Furthermore, unconjugated dendrimers mainly adhere to biological membranes after intravenous administration.[28] Collectively, the data therefore suggest that amine-terminated dendrimers have limited usefulness as drug delivery vectors.[34]

In contrast to unconjugated dendrimers, surface-modified dendrimers show more favorable toxicological and biopharmaceutical properties and, hence, are more suitable candidates as drug delivery vectors. Acetylation of cationic sites on large multifunctional PAMAM dendrimers increases their biological compatibility.[35] Dendrimers possessing polyanionic surfaces also exhibit greatly reduced tissue adhesion and cytotoxicity, with extended circulation times in blood when compared to unconjugated dendrimers.[31,34] However, sulfonate-decorated polylysine dendrimers have been shown to be opsonized, resulting in distribution toward organs of the RES, such as the liver and spleen.[31] Uptake into these organs can be greatly reduced by conjugation to a biologically inert, water-soluble polymer such as polyethylene glycol (PEG).[36] PEG chains (which typically range in size from 0.2 to 40 kDa) increase both the hydrophilicity and size of dendrimers. Small PEGylated dendrimers are excreted mainly via the urine, while larger PEGylated dendrimers may circulate for an extended period in blood and are readily transported into the lymphatic system.[34,36−39]

For dendrimers possessing both drug and PEG molecules bound to the surface, partial surface PEGylation can mask surface-bound drug from *in vivo* binding sites, potentially reducing receptor binding affinity.[40] However, it has also been reported that, in the case of PEGylated proteins, the increased circulation times facilitated by the attachment of PEG also increase exposure to the receptor, thereby potentially improving *in vivo* binding efficacy.[41,42] Hence, surface PEGylation is an attractive and increasingly employed strategy to increase the circulation times of dendrimers and to enable more prolonged retention of associated drug in plasma as a carrier−drug complex.

To this end, the EPR effect is dependent on nanomaterials having long circulating characteristics that maximize the chances of the particle extravasating across the tumor vasculature. Dendrimers are small compared to typical drug carriers, and hence although PEGylation can reduce protein binding and removal by the reticuloendothelial system, this is not sufficient to ensure long circulation behavior. PEGylated dendrimers with low molecular weight have been shown to be rapidly cleared intact via the urine if their size is sufficiently small to also allow renal filtration (Figure 3).[36] Hence, long

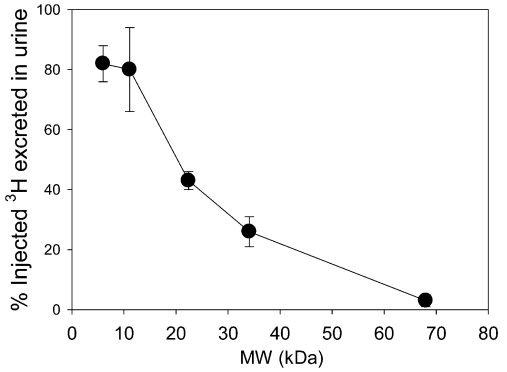

Figure 3. Correlation between molecular weight and the proportion of an injected dose of ^{3}H-labeled PEGylated polylysine dendrimers excreted in urine in rats administered 5 mg/kg dendrimer intravenously. Data represent mean ± SD (n = 3−5). Reprinted with permission from ref 36. Copyright 2008 American Chemical Society.

circulating characteristics will only be achieved by also increasing the molecular weight of the dendrimer. Increasing the overall size of the dendrimer can be achieved in two main ways. Size may be increased by growing the size of the initial core or increasing the number of generations in the scaffold, where each generation results in an increase of approximately 1 nm in diameter.[43] Alternatively the attachment of large bulky surface groups may also be employed to increase the overall size of the dendrimer. In this latter case, the surface conjugation of polymers (e.g., PEG) or lipid chains can substantially

Table 1. Dendritic Systems Containing Chemotherapeutic Drug via Noncovalent and Covalent Interactions

noncovalent drug association			covalent drug association			
dendrimer	*in vivo* evaluation	ref	dendrimer	linker	*in vivo* evaluation	ref
			Methotrexate			
PAMAM	no	50,103,104	PAMAM	amide	no	105,106
PAMAM	yes	60	PAMAM	amide	yes	7,60
polyether-*co*-polyester	no	58,59	PAMAM	ester	yes	8,61−65,107−109
melamine	yes	110	polylysine	amide	yes	40
			polylysine	peptide	yes	66
			polyglycerol	peptide	no	75
			Doxorubicin			
PAMAM	no	50,111	PAMAM	amide	no	112−114
PAMAM	yes	67,70	PAMAM	hydrazone	no	112,113
PPI	yes	68,69	PAMAM	ester	no	115
			PAMAM	*cis*-aconityl	yes	60,76,116
			polylysine	hydrazone	yes	74
			polyester	hydrazone	yes	30,37,71
			polyester-polyamide	hydrazone	yes	117
			polyglutamic acid	hydrazone	no	73
			polyglycerol	peptide	no	75
			polylysine octasilsesquioxane	disulfide spacer	no	118
			Paclitaxel			
PAMAM	no	119	PAMAM	ester	no	35,80
polyglycerol	no	78,79	triazine	ester	no	120
triazine	yes	121	triazine	ester	yes	81,82
			5-Fluorouracil			
PAMAM	no	85,86				
PAMAM	yes	51,83−85				
PAMAM	no	122				
			Camptothecins			
PAMAM	yes	48,91	PAMAM	ester	no	123
PAMAM	no	93	PAMAM	peptide	no	97
polyester	no	92	polylysine	amino acid	yes	124
			Saporins			
PAMAM	no	113				
			Chloroquine Phosphate			
polylysine-galactose	yes	125				

increase hydrodynamic radii as well as dendrimer molecular weight.[28,31,34,36,39,44]

Dendrimers can therefore be designed to provide optimal passive and active targeting toward solid and disseminated cancers, while avoiding accumulation in noncancerous organs and enabling clearance of the dendrimer scaffold once drug has been delivered to cancer cells. This review, however, is not focused on optimizing the design of dendrimers for tumor-targeting, but rather, in identifying the best method of associating chemotherapeutic drugs with the dendritic carrier such that drug delivery toward the cancer is optimized, while exposure of noncancerous cells to the toxic effects of the free drug is minimized. The aim of the next section is therefore to describe the different means by which chemotherapeutic drugs can be associated with dendrimers, and the advantages and disadvantages of each approach.

4. DENDRIMERS AS CHEMOTHERAPEUTIC DRUG CARRIERS

As mentioned briefly above, drugs may be associated with dendrimers either by noncovalent entrapment via hydrogen bonding, hydrophobic or ionic interactions or by covalent attachment directly to the surface via a labile linkage tailored to release drug in a predicted and controlled fashion. The efficacy of any drug when covalently attached with a nonlabile drug linker to the dendrimer scaffold is often low due to poor interaction with their cellular targets and/or reduced propensity for internalization into cellular compartments. Each of the approaches to drug attachment have their own advantages and disadvantages, but ultimately the ideal system for any given drug is one that enables sufficient drug loading, extended circulation, improved tumor targeting and controlled release of drug, as well as little or no toxicity from the carrier or its byproduct. The number of papers published in the past few years describing studies of dendrimers as carriers for anticancer drugs has grown rapidly. Literature references detailing dendrimers that have been generated as tumor-specific drug delivery systems are highlighted in Table 1.

4.1. General Aspects of Noncovalent Drug Encapsulation in Dendrimers. PAMAM and PPI-based dendrimers have been extensively investigated for their capacity to entrap hydrophobic guest molecules within dendritic structures. Drug entrapment in these structures is favored due to the interior structure of the dendrimers facilitating both hydrophobic encapsulation and hydrogen bonding with tertiary amines.[45] The primary mechanism of drug solubilization within the dendrimer structure, however, is via electrostatic interactions with charged surface amines and inner tertiary nitrogens.[46] For

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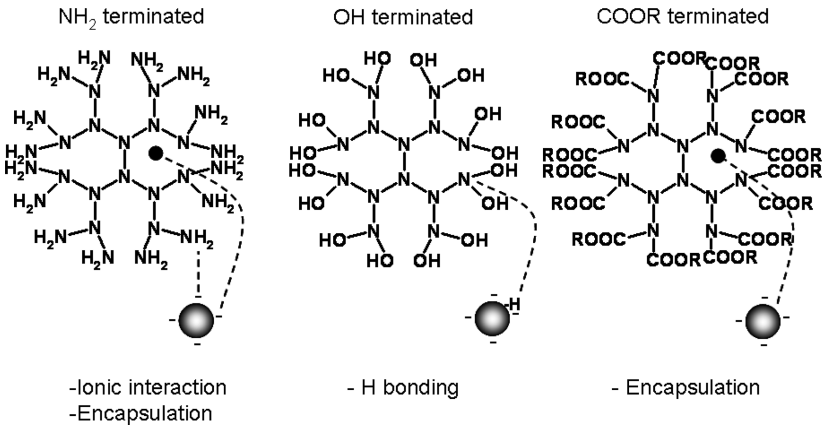

Figure 4. Mechanisms of noncovalent association of weakly acidic, hydrophobic drugs in PAMAM dendrimers containing different surface functional groups.[49].

this reason, the encapsulation efficiency of drug molecules is influenced by dendrimer size, surface structure, solvent pH and functionality of the drug molecule. In general, higher generation dendrimers show improved capacity for drug solubilization compared to lower generation dendrimers due to the larger void volume available for drug encapsulation,[47] and enhanced electrostatic interactions with increasing dendrimer generation.[46,48] Hence, for some drug–dendrimer systems that rely heavily on ionic mechanisms of drug encapsulation, a compromise needs to be reached between the dendrimer size, which promotes maximal drug entrapment, and reduced drug "leakiness".

The surface structure can also influence the mechanisms available for drug solubilization. As illustrated in Figure 4, amine-terminated dendrimers entrap weakly acidic drugs mainly via electrostatic interactions with both terminal and interior amine groups as well as via hydrophobic encapsulation. Hydroxy-terminated dendrimers solubilize drug (such as methotrexate) mainly via weak hydrogen bonding, while ester-containing surfaces result in hydrophobic encapsulation (Figure 4).[49] Some authors have reported that attachment of PEG chains to the dendrimer surface can also increase drug encapsulation by increasing the overall volume for hydrophobic drugs to be encapsulated via hydrogen bonding and electrostatic interactions.[50,51] The size of the PEG chain, however, must be considered in order to achieve optimal drug solubilization. On account of their greater rigidity, longer PEG chains have an apparently increased capacity to solubilize more drug than shorter PEG chains.[50] However, PEG chains that are larger than approximately 5000 Da can similarly reduce drug encapsulation. This is believed to be due to the formation of agglomerates that are generated as a result of the much longer PEG chains forming large structures that fill the interior space of the dendrimer, reducing the volume available for drug encapsulation.[52]

The nature of the dispersing medium can also have a significant impact on drug encapsulation and stability within the dendrimer. Solubilization and release studies conducted on a number of model drugs have revealed that the pH, the ionic strength and the presence or absence of drug binding proteins in the aqueous medium can have a significant impact on drug entrapment. The pH of the medium can influence the extent of ionization, both of the guest molecules and of the dendritic amines.[53] Typically, solution pH conditions that limit drug encapsulation will also increase drug release from the dendrimer (Figure 5). Increasing the tonicity of the medium can also promote competition with drug for electrostatic

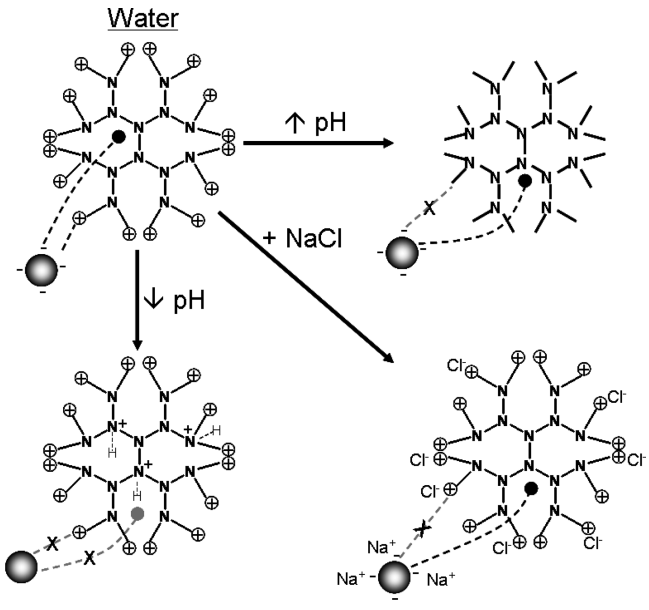

Figure 5. Effect of pH and salt on hydrophobic encapsulation and electrostatic interactions of weakly acidic, hydrophobic drugs in PAMAM dendrimers that terminate with primary amines. Inhibition of specific solubilization pathways is indicated by a cross.

binding sites on the dendrimer, thus increasing the release of drug (Figure 5).[50,51,54] This effect is more substantial for drugs such as methotrexate and many of the nonsteroidal anti-inflammatory drugs that are entrapped within dendrimers primarily via ionic interactions. It is also for this reason that many investigators have observed good stability of dendrimer–drug inclusion complexes in water but rapid release in buffered saline. Finally, the presence of albumin can influence the stability of drug–dendrimer inclusion complexes by encapsulating either the drug or the dendrimer via electrostatic interactions with anionic sites in the protein structure or by solubilization within the hydrophobic interior.[55–57] This highlights the importance of investigating not only the stability of drug–dendrimer inclusion complexes in model media, but also in biological systems, since pH, tonicity, dilution effects, drug binding proteins and the presence of biological

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Table 2. Summary of Advantages and Disadvantages of Drug Entrapment and Covalent Association of Chemotherapeutic Drugs with Dendrimers as a Means to Improving the Antitumor Efficacy of the Drugs

entrapment		covalent association	
advantage	disadvantage	advantage	disadvantage
Methotrexate			
good entrapment efficiency	burst release	good conjugate stability	potential biodistribution resembling MTX interaction with receptors
liberation of unmodified drug	little *in vivo* therapeutic benefit	slow drug liberation	potential liberation of less active modified drug
	little pharmacokinetic benefit	improved tumor biodistribution	
		improved therapeutic efficacy	
Doxorubicin			
improved oral bioavailability	burst release	good conjugate stability	rate of drug release with some linkers is too slow to improve therapy (with acid-labile linkers)
slower drug release after initial burst release	rapid clearance of drug from plasma	reduced systemic drug toxicity	
potential acid mediated drug release		improved therapeutic efficacy	
increased circulation time of drug		improved tumor biodistribution	
improved tumor distribution			
Paclitaxel			
improved aqueous solubility of drug		improved drug solubility	no improvement in tumor biodistribution reported
steady rate of drug release		reduced systemic drug toxicity	potential uptake into reticuloendothelial organs
		good antitumor efficacy	
5-Fluorouracil			
increased circulation time of drug		not determined	not determined
improved tumor biodistribution			
reduced uptake of drug in drug sensitive organs			
improved therapeutic efficacy for more stable systems			
Camptothecins			
improved aqueous drug solubility	drug solubilization dependent on ionization of drug and dendrimer functionality	good complex stability	
potential for acid mediated drug release	no *in vivo* evidence of therapeutic benefit	improved tumor biodistribution	
		improved therapeutic efficacy	

membranes can all influence the stability of drug encapsulated within a dendrimer.

The aforementioned influence of solution conditions is an important factor in the delivery of anticancer drugs to tumors since long-term stability of the drug–dendrimer complex is required to achieve delivery of drug to the tumor while avoiding systemic exposure of drug that may cause dose-limiting toxic side effects. The effect of pH, in particular, can also become important when aiming to deliver drug to acidic tumors, wherein drug release may be either promoted or reduced by changing the degree of ionization of both drug and dendrimer. Table 1 highlights the major studies where chemotherapeutic drugs have been encapsulated within dendrimers as a means of improving drug delivery toward tumors. In general, PAMAM dendrimers tend to be favored for the entrapment of anticancer drugs over other dendrimers, although this may in part be explained by the increased commercial availability of these dendrimers, and hence their use in *in vitro* studies, rather than conferring a particular advantage in drug encapsulation. The following section summarizes the reported encapsulation and release behavior of the frequently studied anticancer compounds, and discusses the benefits and drawbacks to encapsulating these drugs into dendrimers to provide an improvement in chemotherapy. Table 2 contains a summary of the general advantages and disadvantages of the noncovalent and conjugation approaches for forming drug–dendrimer systems for the major reported anticancer drugs (methotrexate, paclitaxel, doxorubicin, 5-fluorouracil and camptothecin).

4.2. General Aspects of Covalent Conjugation of Chemotherapeutic Drugs to Dendrimers. Chemotherapeutic drugs entrapped within a dendrimer carrier are generally rapidly released in biologically relevant fluids, and hence there has, in general, been little *in vivo* evidence of therapeutic benefit for these systems. However, chemotherapeutic drugs may also be covalently bound to the surface of a dendrimer via labile chemical linkages as an alternative to solubilization or electrostatic association. There are a number of potential chemical linkers that are cleavable by a physiological trigger

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within the tumor tissues. Disulfide, peptide or ester linkers are common examples of linkers that can be nonspecifically cleaved *in vivo,* relying upon tumor-specific accumulation of the drug−dendrimer construct to enable tumor-selective toxicity of the drug. For example, carboxyesters may be nonspecifically cleaved by circulating carboxyesterase in plasma. Linkers may also be designed to be more selectively cleaved by triggers specific to the tumor microenvironment to reduce nonspecific drug liberation in healthy tissues. Utilizing linkers that are selectively cleavable by tumor-specific enzymes and physicochemical characteristics is expected to provide an advantage to the use of less specific linkers by providing better control over the site of drug release and exposure of normal tissues and blood to the cytotoxic drug.

Specifically, the choice of covalent linker is largely informed by the required selectivity and environment in which drug should be released. The use of dendrimers in the delivery of drugs to solid tumors, for instance, takes advantage of the EPR effect to allow the tumor-specific accumulation of dendrimer−drug construct. Thus, the use of a drug linker that enables the release of drug within the extracellular environment of the tumor (such as a pH or matrix metalloproteinase cleavable linker) is a simplistic and reliable way of delivering active drug specifically to a tumor. This approach, however, is highly dependent upon the linker being sufficiently labile in the presence of the extracellular trigger to allow the release of a cytotoxic amount of drug. As an example, most investigators use acid-labile hydrazones or ortho-ester functions to enable drug liberation within the acidic interstitium of solid tumors. This is probably the simplest way of enabling drug release within the tumor since it enables liberation of intact, unmodified drug via hydrolytic cleavage. In this case, however, the hydrazone or ester linker employed must be chosen very carefully since different linker structures have different susceptibilities to hydrolysis at both acidic and neutral pH, which will influence the rate of drug liberation and the antitumor efficacy of the liberated drug. This will be discussed in more detail in a later section.

Alternatively, peptide-based linkers may provide better tumor selective drug liberation, particularly for peptides which are substrates for enzymes that are only constitutively expressed at very low levels elsewhere in the body. A downside to this approach, however, is that again, the cleaved product needs to be sufficiently cytotoxic in order to kill the cancer cells. As an example, Chau and colleagues demonstrated the use of the matrix metalloproteinase 2 and 9 specific peptide, PVGLIG, to conjugate MTX to a dextran carrier. The liberated PVG-modified MTX displayed approximately 10-fold lower *in vitro* cytotoxicity when compared to MTX alone.[21,22] However, in this case, the increased tumor delivery of the MTX−dextran construct overcame this reduced cytotoxicity, resulting in 80% inhibition of HT-1080 tumor growth compared to PBS-dosed mice. Additionally, an equimolar dose of free MTX failed to inhibit tumor growth.

On the other hand, drug conjugation to dendrimers may be via linkers designed to release drug intracellularly in endocytic vesicles. Cathepsin B-cleavable linkers or pH-labile linkers are more labile in the lower pH environment of a lysoendosome (pH 5−6) when compared to the extracellular fluid (pH 5.7−7.8) within a solid tumor. The downside to this approach, however, is that it relies heavily upon the specific cell binding and endocytosis of the dendrimer, often requiring a high surface loading of active targeting ligands that may also recognize other nontumor binding sites as discussed in more detail later.

Finally, amide bonds are generally considered to be relatively stable *in vivo*. Biologically stable amide coupling of drug molecules to dendrimers has been utilized experimentally to enable the investigation of the pharmacokinetic behavior of intact drug−dendrimer conjugates, avoiding the confounding issue of drug liberation, and thereby understanding of the consequences of replacement of surface PEG with hydrophobic drug.[40]

In the following sections and in Table 1, the current literature on the release rates and *in vivo* behavior of covalently linked chemotherapeutic drug−dendrimer conjugates is collated and examined by drug. Table 2 also summarizes the advantages and disadvantages of covalent conjugation of chemotherapeutic drugs.

4.3. Methotrexate (MTX). Methotrexate is an inhibitor of intracellular dihydrofolate reductase that binds to folate and reduced folate carriers. MTX exhibits dose-limiting hepatotoxicity in the clinical setting and therefore cannot be administered to patients with liver disease, or patients on other forms of hepatotoxic medication. Conjugation to or association with nanosized carriers is expected to provide advantage over the administration of free drug by avoiding hepatic accumulation and improving systemic delivery toward solid tumors.

Dhanikula and colleagues examined the encapsulation of MTX in second generation (G2) polyester *co*-polyether dendrimers containing a polyethylene oxide core and branching units composed of gallic acid, dihydroxy benzoic acid (DHBA) or bis(hydroxyl methyl) butyric acid (BHBA).[58] MTX was found to be encapsulated via hydrophobic interactions with the aromatic rings of the gallic acid- and DHBA-based dendrimers and by electrostatic interactions between the amine groups of the dendrimer and the carboxyl groups of the drug. The encapsulation efficiency was reduced dramatically in BHBA dendrimers on account of reduced capacity for hydrophobic entrapment. Burst release of drug from BHBA dendrimers was more extensive when compared with the other dendritic systems in pH 7.4 buffer. All three systems, however, showed burst release of approximately 60−80% of encapsulated MTX in buffered saline over 6 h.

In a later study by the same group, glucosamine was conjugated to the surface of the MTX−dendrimers to improve internalization in rat glioma cells.[59] The targeted dendrimer showed improved *in vitro* cytotoxic efficacy when compared to free drug, particularly in the methotrexate-resistant cell line U37 MG. Increased permeability through the blood brain barrier was suggested for glucosamine-targeted dendrimers in an *in vitro* model, but no *in vivo* efficacy or biodistribution data were reported.

Jiang and colleagues further demonstrated that increasing the encapsulation of MTX within a G4 PAMAM dendrimer by increasing the degree of surface PEGylation had little impact on MTX release kinetics.[60] MTX was rapidly released from all dendrimers in an isotonic buffer, with up to 75% released over 2 h. This resulted in the dendrimers displaying equivalent *in vitro* cytotoxicity and *in vivo* pharmacokinetic profiles to that of free MTX. Interestingly, however, *in vivo*, a PEGylated dendrimer displayed improved antitumor efficacy and body weight profiles in a mouse sarcoma S-180 tumor model when compared to a non-PEGylated MTX-complexed dendrimer. However, the PEGylated dendrimer did not display signifi-

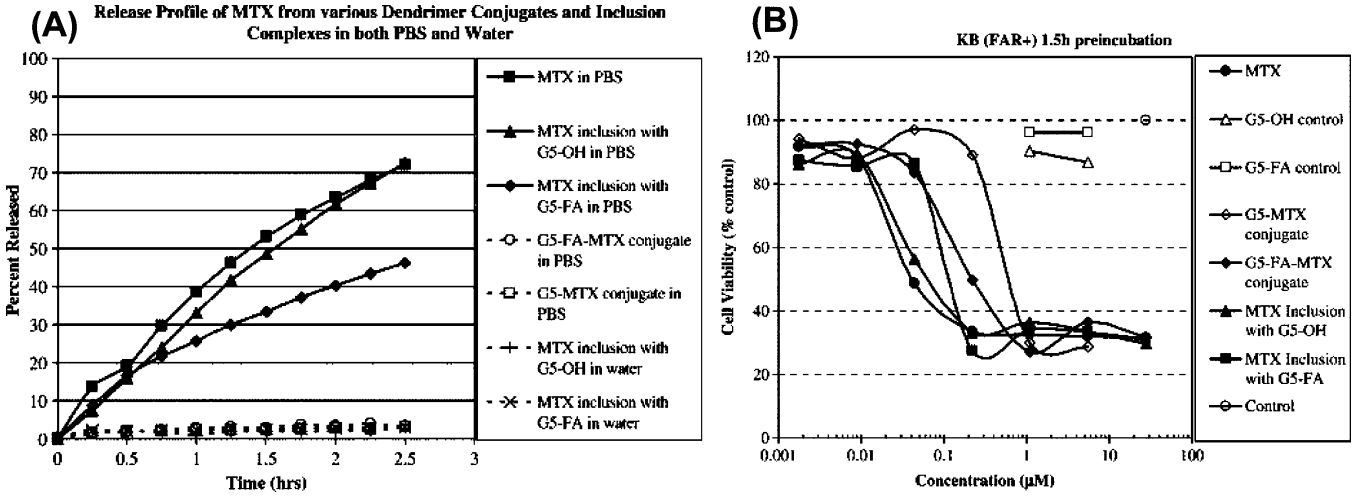

Figure 6. Release profiles of methotrexate (MTX) from G5 PAMAM dendrimers encapsulating MTX or conjugated with MTX in water or phosphate-buffered saline (A). Dendrimers were either surface modified with hydroxyl functionality (G5-OH) or conjugated with folic acid (G5-FA). Cytotoxicity of MTX and dendrimer against KB cells overexpressing folate receptors (FAR+) (B). Cells were incubated with MTX alone, MTX-free dendrimers (G5-OH and G5-FA), media alone (control), MTX conjugated dendrimers (G5-MTX and G5-FA-MTX) or dendrimers encapsulating MTX (MTX inclusion with G5-OH or G5-FA) for 1.5 h prior to replacement of the media with drug free media and further incubation for 72 h. Reprinted with permission from ref 61. Copyright 2005 Elsevier B.V.

cantly improved therapeutic benefit when compared to the administration of free MTX.

The literature therefore suggests that, in general, encapsulation of MTX within the dendrimer scaffold is an inefficient approach to improving the tumor disposition of the drug, since this method of association results in burst release of methotrexate *in vitro* and *in vivo*. This subsequently leads to rapid clearance of methotrexate from plasma and likely limits delivery of the drug to the target tumors. In contrast, covalent conjugation of MTX to the dendrimer surface via one of its carboxyl groups can significantly improve the stability of the complex and therefore improve the tumor disposition of the drug. This was elegantly demonstrated in two of the very few literature studies that have directly compared the *in vivo* and *in vitro* benefits of covalent conjugation of drug to noncovalent encapsulation. In the study by Jiang mentioned previously, the *in vitro* and *in vivo* behavior of a series of PEGylated and unPEGylated PAMAM dendrimers containing surface-conjugated or encapsulated MTX was compared.[60] MTX was relatively stable when conjugated to PEGylated PAMAM dendrimers via amide linkages, with less than 5% MTX released over 48 h and significantly lower *in vitro* cytotoxicity when compared to free and dendrimer encapsulated MTX. The MTX-conjugated dendrimer displayed longer plasma exposure when compared to free MTX and the dendrimer encapsulated drug. Interestingly, however, the antitumor efficacy of the MTX-conjugated dendrimer in S-180 tumor-bearing mice was only marginally better than for the dendrimer with entrapped MTX, with reductions in tumor volume of 78 and 66% respectively when compared to saline-treated controls. This result was most likely due to the more rapid liberation of MTX encapsulated in the dendrimer once the dendrimer had distributed toward the tumor. Patri,[61] on the other hand, generated a series of targeted and nontargeted PAMAM dendrimers containing MTX associated via either a pH-labile ester linkage, or via noncovalent entrapment within the structure. The ester-linked MTX dendrimer was found to be stable in physiological buffered saline with negligible conjugated MTX liberated (Figure 6A). As expected, this conjugate displayed reduced *in vitro* cytotoxic efficacy when compared to the free drug, although the *in vitro* cytotoxicity against folate-overexpressing cancer cells was substantially improved when folic acid targeting ligands were employed (Figure 6B). Noncovalent entrapment of MTX, however, resulted in an almost complete burst release of MTX from the structure in buffered saline within 2.5 h (Figure 6A), resulting in similar *in vitro* cytotoxic efficacy when compared to the free drug (Figure 6B). The burst release was minimized somewhat by inclusion of folic acid, possibly due to increased hydrophobic interactions between the dendrimer and drug. Since the main cytotoxic action of methotrexate is mediated by its ability to inhibit folate-dependent pathways in DNA replication, many investigators have used folic acid-targeted dendrimers to improve the delivery of MTX into folate receptor overexpressing cancers. Although the study by Patri suggests that the folic acid targeting approach can have the added benefit of improving the stability of MTX−dendrimer complexes *in vivo*, burst release of drug is still a big limitation in the use of dendrimers encapsulating MTX as tumor delivery systems. In addition, this study also highlights the importance of generating systems that not only prevent drug leakage in the systemic circulation but also liberate drug rapidly once biodistributed into the tumor.

In further support of the use of covalently conjugated MTX dendrimers as tumor delivery systems, several other studies have demonstrated *in vivo* evidence of improved tumor disposition of drug as well as antitumor activity when compared to methotrexate alone. Most of these studies have utilized G5 PAMAM dendrimers as the dendrimer carriers. In one such study, MTX was conjugated to a folic acid−PAMAM dendrimer via a hydrolyzable ester linker to facilitate endocytosis of MTX−dendrimer followed by acid-mediated intracellular liberation of drug. This construct resulted in reduced *in vitro* cytotoxic efficacy against cultured KB cells when compared to free MTX, presumably due to slower intracellular delivery of MTX.[62] Despite the fact that approximately half of the attached drug was expected to have been released *in vitro* after 7 days, details of this investigation were not reported. However, despite the relatively rapid

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clearance of the dendrimer from blood via the kidneys, the folic acid-targeted conjugate showed 3-fold higher accumulation in KB tumor tissue when compared to the nontargeted dendrimer 7 days after administration.[8] This construct provided an increase in the *in vivo* antitumor efficacy of the targeted construct in KB tumor-bearing mice when compared to an equimolar dose of free MTX.[8,35,63−65] The targeted construct, however, was also distributed approximately twice as avidly into the liver as the untargeted system, one of the main sites of MTX toxicity. This suggested that although the dendrimer conjugate increased the tumor distribution of MTX and the antitumor efficacy, conjugation of MTX to folic acid-targeted dendrimers may not significantly reduce hepatotoxicity of the bound drug on account of the extensive accumulation in the liver. Unfortunately, markers of hepatic and systemic toxicity were not reported in these studies.

The tumor disposition and antitumor efficacy of MTX-conjugated biodegradable polylysine dendrimers, which have not employed the folic acid targeting strategy, have also been investigated. In this work, conjugation of α-carboxyl protected (O-tBu) MTX via a stable amide linker to 50% of the surface amines, with PEG (570−2200 Da) conjugated to the remaining amines, provided extended plasma circulation times.[40] A G5 dendrimer constructed with a mixed drug−PEG1100 surface administered iv to nude mice bearing subcutaneously implanted HT1080 tumors displayed equivalent accumulation in tumor and spleen, similar to that of the previously mentioned targeted PAMAM dendrimer. Other organs, however, showed accumulation of dendrimer at levels less than 30% of that deposited in tumor and spleen. This suggests that targeting ligands may not be required to obtain good targeting of MTX to solid tumors, although they may be useful in promoting uptake of the drug−dendrimer complex selectively into cancer cells.

In a follow-up study, MTX was conjugated to a PEGylated G5 polylysine dendrimer via a hexapeptide linker (PVGLIG) designed to be cleaved in the presence of matrix metalloproteinases 2 and 9.[66] Although the MTX−PVGLIG conjugated dendrimer was cleared from plasma via the liver within 4 h (proposed to be due to projection of MTX from the PEG layer), the OtBu modified MTX−PVGLIG dendrimer displayed prolonged plasma exposure with a terminal half-life of approximately 1 day (Figure 7). Although the mechanism by which the MTX−PVGLIG construct was cleared via the liver was not identified, it was proposed to be due either to the increased exposure of MTX to folate or reduced folate carriers in the liver or from exposure of the anionic charge of MTX to plasma opsonins followed by uptake by liver macrophages. Interestingly, although the MMP-mediated cleavage of the PVGLIG linker liberates a peptide-modified drug (OtBu-MTX−PVG) that displays 10-fold lower *in vitro* cytotoxicity than MTX alone, this was not reflected by the *in vivo* antitumor efficacy studies in HT1080 bearing mice. On account of the improved biodistribution of MTX into the tumor, the construct slowed the growth of HT1080 tumors more avidly than administration of molar equivalents of MTX. The MTX−PVGLIG construct that was cleared via the liver, however, displayed no reduction in tumor growth when compared to saline-dosed control mice. The results of this study therefore suggest that although the conjugation of drugs to the surface of a dendrimer may improve tumor targeting and therapeutic efficacy when compared to free MTX, the choice of drug linker and *in vivo* targets for the attached drug must be carefully considered.

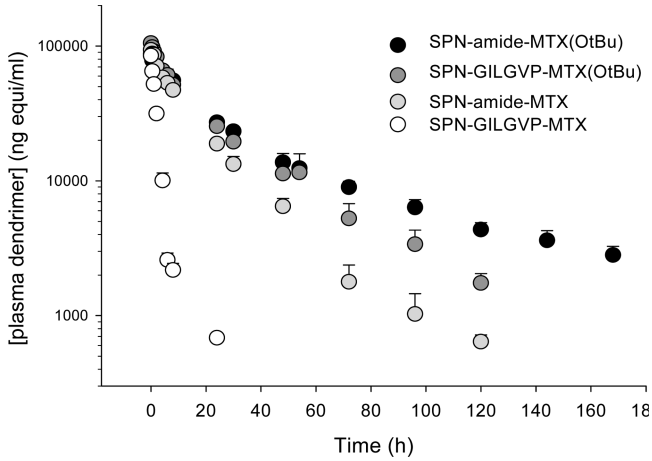

Figure 7. Plasma concentration−time profile for ^{3}H-labeled G5 polylysine dendrimers containing PEG1100 at terminal α-amines and methotrexate (MTX) or α-carbonyl conjugated OtBu-methotrexate (MTX(OtBu)) at terminal ε-amines dosed iv to rats at 5 mg/kg. The fifth generation was composed of either L-lysine or a symmetrical analogue of lysine (succinimyldipropyldiamine, SPN). MTX was conjugated via either amide linkages or matrix metalloproteinase 2 and 9 cleavable PVGLIG hexapeptide linkers. The data is represented as mean ± SD (n = 3). Reprinted with permission from ref 66. Copyright 2010 American Chemical Society.

4.4. Doxorubicin (DOX). Doxorubicin is an anthracycline antibiotic used in the treatment of a range of solid tumors, lymphomas and leukemias. DOX, however, has a very short half-life in plasma on account of its rapid cellular binding and trafficking to the nucleus, resulting in widespread biodistribution. The main dose-limiting side effect of DOX treatment is irreversible cardiotoxicity such that the maximum cumulative dose of DOX throughout a patient's lifetime is limited to 550 mg/m^2. The systemic toxicity of the drug has also been linked to maximal plasma concentrations achieved after iv administration, although the bioavailability of the drug after oral administration is too low to be therapeutically useful. Therefore, encapsulating DOX within drug carriers, including dendrimers, has been proposed as a means to increase DOX bioavailability after both oral and intravenous administration. To this end, Ke and colleagues encapsulated DOX within a G3 PAMAM dendrimer and examined drug leakage from the construct.[67] They found that approximately 50% of entrapped DOX was released within 2 h after solubilization in buffer at pH 7.4, and that the level of drug release plateaued to approximately 70% over 24 h. Interestingly, despite relatively rapid release of drug from the dendrimer, increased oral bioavailability of the drug was still observed when compared to the administration of free drug. To date, however, no evidence of improved *in vivo* antitumor efficacy has been reported following oral administration of a drug entrapped in a dendrimer.

Agrawal also observed approximately 70% release of DOX from a G5 dextran-conjugated PPI dendrimer over 24 h at pH 7.4.[68] Buffer tonicity and the presence of albumin had no effect on drug release. Encapsulated drug was released quantitatively and more rapidly over 8 h in a more acidic pH 6.4 buffer and was suggested to be due to increased protonation of amines within the dendrimer structure. The preliminary data therefore suggested likely stability in plasma, but drug liberation within the acidic environment of solid tumors, and hence that this

construct would represent a useful *in vivo* tumor delivery system. The dendrimer also showed equivalent *in vitro* cytotoxicity on A549 cells when compared to free drug, although this may have been due to acidification of the cell culture media and complete liberation of free DOX. Although the circulation time of total DOX in rats after intravenous administration was increased, the clearance of DOX was still relatively rapid, likely on account of both rapid clearance of the intact dendrimer and gradual leakage of the drug from the complex. The concentration of DOX equivalents, however, was higher in tumor tissue 24 h after administration of the encapsulated drug when compared to administration of free drug. This suggests that although the system displayed relatively rapid DOX clearance after iv administration, increased delivery of drug to solid tumors was still observed over more extended times relative to administration of free drug, presumably on account of the increased circulation time and reduced vascular binding of DOX.

Later work by the same group showed that varying the surface functionality of the dendrimer may alter the encapsulation efficiency as well as DOX release and stability profiles. For example, a folic acid-conjugated G5 PPI dendrimer showed over 2-fold improved DOX encapsulation, although release was more rapid, with over 80% of DOX released within the first 24 h in phosphate buffered saline at both pH 7.4 and 6.4.[69] The surface conjugation with folic acid did, however, result in a 4-fold reduction in hemolytic toxicity of the dendrimer due to shielding of its cationic surface charge. No *in vivo* efficacy data were reported.

In general, therefore, the encapsulation of DOX within dendrimers results in rapid drug release, limiting the therapeutic utility of this method of drug association with dendrimer carriers. A comment on the therapeutic usefulness of DOX encapsulated in dendrimers cannot reasonably be made. However, given the lack of antitumor efficacy data reported for these constructs, more recently Han[70] generated a more stable DOX release profile from a PEGylated G5 PAMAM dendrimer in pH 7.4 buffer by initially complexing DOX to a codelivered apoptosis-inducing gene (TRAIL) before encapsulation in the dendrimer. By initially complexing DOX with the gene, the liberation of DOX from the dendrimer was more dependent on the liberation of the gene from the dendrimer. This had the effect of reducing the dependence of DOX release on electrostatic interactions with the scaffold. Specifically, only 20% of DOX was released from the dendrimer over 120 h using this approach. Although this approach resulted in greater DOX stability within the dendrimer, only the dendrimer system containing a cancer targeting ligand displayed improved antitumor efficacy when compared to administration of DOX alone. Reduced cytotoxicity of the DOX−DNA complex or reduced rate of DOX liberation may have been responsible for the poor efficacy outcome. This study does, however, highlight the potential advantages of prior reversible modification of cytotoxic drugs before encapsulation within dendrimers to change the *in vivo* drug release kinetics from the system.

In contrast to DOX encapsulated in dendrimers, which have not been well characterized for their potential benefit *in vivo*, there is good literature evidence to suggest that conjugation of DOX via acid-labile linkers to the dendrimer surface can improve antitumor efficacy and/or reduce systemic toxicity of DOX. This is, however, dependent on the stability of the linker, which can result in either too rapid or too slow DOX liberation to demonstrate therapeutic advantage over administration of DOX alone. For example, Szoka and colleagues[71] previously reported good drug stability at pH 7.4 and drug lability (∼40% over 24 h) from a PEGylated polyester dendrimer conjugated with DOX via a hydrazone carboxylate linker. At physiological pH the conjugate showed reduced *in vitro* toxicity and nuclear accumulation when compared to the free drug. However, the group later reported inefficient *in vivo* antitumor efficacy, in spite of the apparent lability of the linker in an acidic environment and stability at neutral pH. Further investigation revealed that this was likely due to an inability of the previous analytical method to detect the rapid liberation of a DOX cyclization product.[72] Hence release of the DOX derivative was likely to occur prior to reaching the tumor site, due to increased relative lability of the hydrazone carboxylate linker when compared to acyl hydrazone architectures.

In a more recent study, a similar structure containing DOX conjugated via an acyl hydrazone linker showed enhanced anticancer efficacy against a C57 mouse tumor model when compared to free DOX.[30] In addition, administration of 20 mg DOX equivalents per kg body weight as drug-conjugated dendrimer produced no deaths in the mice due to systemic toxicity or tumor growth, whereas no cures were observed in mice administered the maximal tolerated dose of free DOX (6 mg/kg) when compared to an untreated control group.

In a later study, conjugation of DOX to a G4 PEGylated PAMAM dendrimer via a hydrazone linker resulted in ∼10% DOX release at pH 7.4 and ∼80% release at pH 5.5 after a 24 h incubation in buffered saline. *In vitro* cytotoxicity investigations revealed a 10-fold lower cytotoxic efficacy of the DOX-conjugated dendrimer when compared to free DOX. The reduced *in vitro* cytotoxicity of the DOX-conjugated dendrimer when compared to free DOX can be overcome, however, via the use of active cancer-targeting ligands. For example, a study where DOX was conjugated to a biotin-poly(L-glutamic acid) dendrimer via a hydrazone linkage reported similar *in vitro* release profiles to that described above, however cytotoxicity against HeLa cell lines was comparable to the free drug, likely due to receptor-mediated endocytosis which may overcome DOX resistance by internalizing the drug via alternative mechanisms.[73]

In a similar study, DOX was conjugated to two G5 PEGylated polylysine dendrimers bearing different monomers in the outer generation via a hydrazone linker, and displayed long plasma circulation times and preferential uptake into solid rat Walker 256 tumors via EPR and the spleen.[74] These constructs displayed approximately 10% DOX liberation over 48 h in pH 7.4 buffered saline, but almost complete drug liberation over 48 h at pH 5. As a result, administration of DOX covalently linked to dendrimer showed equivalent *in vivo* anticancer efficacy against rat Walker 256 tumors but reduced evidence of systemic toxicity when compared to equivalent doses of free and liposomal DOX. This suggests that while the exposure of the tumor to DOX may have been too slow to provide benefit in terms of antitumor efficacy, the conjugate was sufficiently stable to limit systemic exposure of DOX, resulting in the capability to administer higher doses of DOX.

Hydrazone linkages appear to be the linker of choice for the covalent attachment of DOX to dendrimer constructs, however several other linkers have also been explored more recently. A maleimide-bearing DOX prodrug conjugated to thiolated dendritic polyglycerol employed the enzymatic cleavage of a peptide linker by cathepsin B, which is overexpressed in several solid tumors.[75] Although the amount of drug released from the

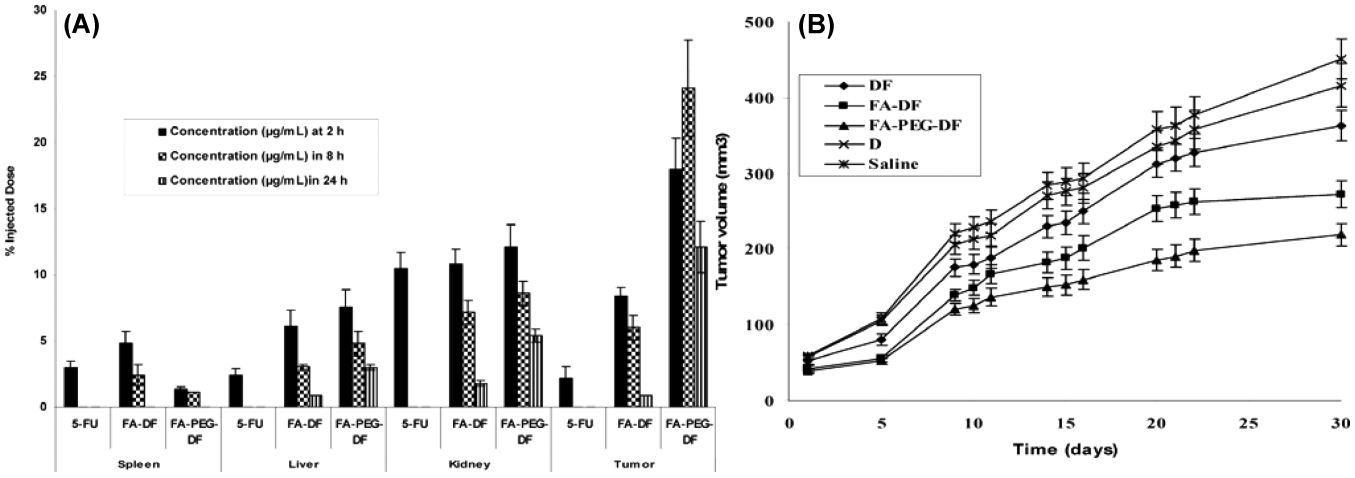

Figure 8. Biodistribution of 5-FU at different time points administered as free drug and dendrimer formulations to Balb/c mice bearing subcutaneously implanted KB tumors (A) (left). Results of antitumor efficacy screening in Balb/c mice bearing subcutaneously implanted KB tumors (B) (right). For antitumor efficacy studies, mice were intravenously administered 12 mg/kg 5-FU equivalents of each formulation 21 and 28 days after injection of tumor cells. G4 PAMAM dendrimers containing entrapped 5-FU were conjugated with PEG−folic acid (FA−PEG−DF) or folic acid alone (FA−DF) or were unconjugated (DF). G4 PAMAM dendrimers not containing drug, PEG or FA are represented as D. Data are represented as mean ± SD (n = 6). Reprinted with permission from ref 84. Copyright 2008 American Chemical Society.

conjugate following incubation at pH 5.0 with cathepsin B was not reported, chromatograms showed effective release of DOX after 2.5 h. Zhu and colleagues[76,77] also examined the effects of PEGylation on the conjugation of DOX via an acid-labile, *cis*-aconityl linker to PEGylated PAMAM dendrimers. Polymer conjugates were stable at pH 7.4−6.6, with <5% released over 96 h, approximately 15% released at pH 5.5 and up to 60% released at pH 4.5. The amount of DOX liberated from the dendrimers was increased with an increase in the extent of PEGylation, however the PEGylation also decreased cellular uptake and *in vitro* cytotoxicity of the constructs. The proportion of DOX liberated from this system, however, was lower than for constructs that employ hydrazone linkers, and may explain the limited improvement in *in vivo* antitumor efficacy observed against B16 melanoma in mice compared to free DOX.

4.5. Paclitaxel. Paclitaxel has a very low aqueous solubility (approximately 0.3 μg/mL) and as a result is formulated for intravenous infusion in Cremophor EL and ethanol or as a nanoparticle with albumin (Abraxane). Many of the commonly reported side effects of iv administered paclitaxel, however, are associated with injection of the excipient Chremophor EL. Association of paclitaxel with a sufficiently soluble drug carrier is therefore ideally required to enable administration of a therapeutically relevant dose which can avoid issues associated with administration of excipients. However, in the preparation of covalent paclitaxel−dendrimer conjugates one must be mindful of the impact of paclitaxel conjugation at the periphery on reduced solubility of the dendrimer carrier.

To this end, Ooya and colleagues[78,79] encapsulated paclitaxel into polyglycerol dendrimers and reported up to 430-fold increase in aqueous drug solubility in a 10% solution of the largest (G5) dendrimer. Increasing the proportion of the G5 dendrimer in the solution to 80% w/w, however, resulted in a 10,000-fold increase in aqueous solubility. Furthermore, *in vitro* drug release studies in buffer and serum showed that paclitaxel was liberated from the G3−G5 dendrimers such that all of the entrapped paclitaxel was liberated from the dendrimer in buffer in 4 days. This suggests that, for paclitaxel, drug entrapment within a dendrimer is a viable option for targeted drug delivery to tumors, although to date, there has been no report of *in vivo* biodistribution or antitumor efficacy studies with a paclitaxel-loaded dendrimer.

Similarly, Khandare increased the solubility of paclitaxel by 10,000-fold by conjugating the drug to a G4 hydroxyl-terminated PAMAM dendrimer via a cleavable ester linker.[80] The conjugate was shown to release approximately 30% of the bound drug over 2 days in a buffered (pH 7.4) solution containing esterase as a hydrolyzing enzyme. *In vitro*, the dendrimer showed a 10-fold increase in cytotoxic efficacy against A2780 carcinoma cells when compared to free paclitaxel. In a similar study, Majoros[35] linked paclitaxel to a G5 folic acid-targeted PAMAM dendrimer and reported similar cytotoxic efficacy against KB cells when compared to free drug. However, the anticancer activity reported was due to the cellular binding and internalization of the dendrimer on account of the folic acid targeting ligands, since cytotoxic efficacy was reduced on a folate receptor deficient cell line.

More recent studies have investigated the novelty of conjugating paclitaxel to triazine dendrimers via hydroxyl group ester linkages. Lim[81] showed that dendrimer-conjugated paclitaxel displayed reduced toxicity *in vivo* when administered to mice at 3 times the maximal tolerated dose of the free drug. However, biodistribution studies revealed a lack of targeting toward PC-3 tumors in mice and high accumulation in reticuloendothelial organs. Cumulative release studies in plasma showed steady release of paclitaxel from the PEGylated construct over 48 h with up to 20% drug release observed in rat plasma and up to 7% in mice.[82] This construct significantly inhibited tumor growth in mice bearing PC-3 xenografts treated over 70 days with comparable antitumor efficacy to Abraxane.

4.6. 5-Fluorouracil (5-FU). 5-FU exerts its chemotherapeutic effects by irreversibly inhibiting the action of thymidylate synthase, preventing the synthesis and incorporation of thymidine into newly forming DNA strands. In addition to intravenous administration to treat systemic cancers, it may also be applied topically to treat certain skin conditions and basal cell skin cancers. Association of 5-FU with dendrimer carriers has therefore been proposed as a means to

limit its systemic exposure after iv administration and reduce the severity of its side effects.

On encapsulation of 5-FU within a G4 or G5 PAMAM dendrimer, drug release has consistently been rapid and complete in water or buffered saline over a period of approximately 24 h.[83−86] The release rate of 5-FU can be slowed, however, down to approximately 60% over 24 h via the conjugation of folic acid or PEG onto the dendrimer surface. This has been demonstrated, for instance, using a PEG5000 conjugated PAMAM dendrimer.[84] However, the rate of 5-FU liberation in biological systems (e.g., after iv administration) is considerably higher than in simple solutions of dendrimer in water.[83] This demonstrates the critical importance of examining the release of drug encapsulated dendrimer in systems that mimic biological fluids, such as buffered saline, serum or isotonic solutions containing albumin.

Nevertheless, Bhadra[51] found that iv administration of 5-FU entrapped in a PAMAM dendrimer increased the plasma residence of the drug and substantially decreased peak plasma concentrations of the free drug when compared to iv administration of drug alone. Singh[84] also reported increased tumor distribution and decreased liver, spleen and kidney distribution of 5-FU when administered as drug entrapped within a PEGylated folic acid targeted dendrimer when compared to administration of the free drug (Figure 8A). Consequently, they reported approximately 50% growth reduction of KB tumors in mice when compared to saline-treated controls, while the administration of the free drug alone had little effect on tumor growth (Figure 8B). This was likely due to the reported increase in the tumor distribution of 5-FU when associated with the dendrimer, compared to administration of drug alone.

4.7. Camptothecins. Camptothecins are naturally derived anticancer agents that inhibit topoisomerase I. However, on account of their generally poor aqueous solubility and unfavorable pharmacokinetic profiles, earlier camptothecins often performed poorly in clinical trials, showing either poor antitumor efficacy or severe hematological toxicity.[87,88] Additionally, camptothecins are not well internalized by cells. Derivatives with greater solubility that display improved pharmacokinetic properties have been produced and marketed, although they show increased systemic side effects on account of the structural modifications required to improve their aqueous solubility.[89,90] Hence, association of camptothecins with polymers to increase their solubility is considered an important therapeutic goal, and several studies have demonstrated that encapsulation of camptothecins within dendrimers achieves several orders of magnitude improvement in the aqueous solubility of drug.[48,91−93]

The capacity of amine-terminated PAMAM dendrimers to solubilize camptothecins is dependent on the functionality interacting with incorporated drug, and the ionization state of the drug molecule. For example, PAMAM dendrimers of generation 4−6 have a reduced capacity to solubilize the lactone form of camptothecin compared to the inactive carboxylate form.[48] The solubilizing capacity is also highly dependent on the concentration and generation of the dendrimer used. For example, for an amine-terminated G4 dendrimer, approximately 25 to 50% of entrapped drug (7-ethyl-10-hydroxy camptothecin) was released in 24 h in pH 7.4 buffer, while at pH 5, 90% of the entrapped drug was released in 30 min. While this study was aimed at examining the potential for PAMAM dendrimers to increase the oral availability of camptothecins, cytotoxicity and cellular uptake studies on Caco-2 cells revealed similar cytotoxicity to the free drug, but increased cellular internalization and permeability through a Caco-2 cell monolayer at high concentrations. There have not been any *in vivo* studies conducted, however, to demonstrate the usefulness of dendrimers to improve the oral bioavailability or tumor disposition/anticancer efficacy of camptothecins. Notably however, *in vivo* studies with other nanocarriers such as polymers and liposomes have been largely unsuccessful or have provided only moderate improvement in chemotherapy when compared to administration of drug alone.[94−96]

Thus far there have been only three studies describing covalently associated camptothecin analogues with a dendrimer. Two of these have employed self-immolative dendrimers, which are discussed in the next section. In the third study, camptothecin−glycine and camptothecin−alanine prodrugs were linked via an ester bond to a polylysine dendrimer decorated with PEGylated (5 kDa PEG) aspartic acid on the surface.[97] These systems displayed greater camptothecin cleavage at pH 7.4 when compared to pH 5, and hydrolysis of the ester bond was independent of the presence or absence of esterases. Thus, the systems generated in this instance did not display tumor selective drug liberation and, rather, antitumor activity was dependent on the increased distribution of drug into the tumor and drug liberation over time, despite the obvious disadvantage of reduced drug release in highly acidic tumors. The glycine−camptothecin system displayed a drug release half-life of 20 h at pH 7.4, however the alanine−camptothecin system was more stable, with a drug release half-life of 200 h. The glycine−camptothecin system displayed a more prolonged circulation time when compared to camptothecin alone (Figure 9a) and also displayed improved tumor distribution of drug and reduced distribution of drug into the lungs liver and spleen (Figure 9b). Consequently, the dendrimer displayed improved antitumor efficacy against C26 and HT-29 carcinomas when compared to administration of camptothecin alone (Figure 9c).

Camptothecins have also been covalently linked to a relatively new class of dendrimers which allow all drug molecules to be simultaneously released via a method involving enzymatic activation of a central retro-Aldol retro-Michael trigger.[98] Development of these types of dendritic drug delivery devices, however, has been limited to low generation dendritic structures, but shows some potential for further development. Thus far, only two attempts have been made to covalently conjugate chemotherapeutic drugs (thus far only camptothecins) to these "self-immolative" dendrimers.[98,99] The triggers used have included linkers that are cleavable by model enzymes such as the catalytic antibody 38C2 and penicillin-G-amidase. In the first of these studies, camptothecin or DOX was conjugated to a G1 dendrimer in a proof of concept study.[99] Attachment of these drugs to the dendrimer dramatically reduced the cytotoxicity of the drugs against Molt-3 cells in culture compared to the free drug. However, upon addition of 38C2 catalytic antibody, the cytotoxic efficacies of the conjugated dendrimers were significantly increased. The dendritic constructs, however, displayed less *in vitro* cytotoxicity when compared to free drug even in the presence of the catalytic trigger. In the second study,[98] G2 dendrimers were PEGylated to increase the solubility of the complex, but enzymatic cleavage still resulted in reduced cytotoxic efficacy when compared to the free drug. Although these studies

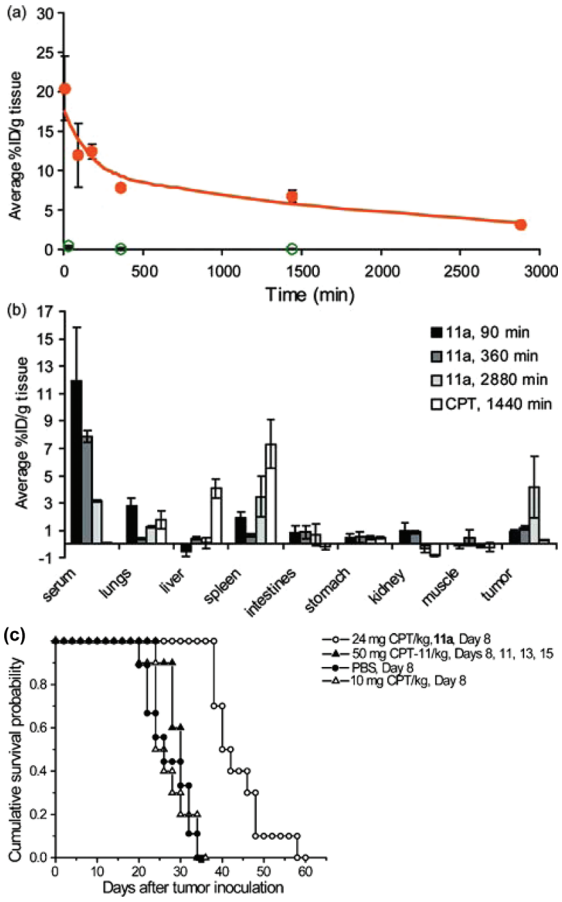

Figure 9. Blood concentration–time profile (a) and organ biodistribution (b) of free camptothecin (CPT, open symbols) and a camptothecin-conjugated PEGylated polylysine dendrimer (**11a**, closed symbols) in Balb/c mice bearing C26 tumors after iv administration of 10 mg/kg camptothecin equivalents. The comparative antitumor efficacy of CPT and CPT-conjugated dendrimer against C26 tumors in mice is shown in (c). Mice were intravenously administered PBS vehicle alone 8 days after sc injection of cells, 10 mg/kg free CPT alone 8 days after injection of cells, 24 mg/kg CPT-conjugated dendrimer 8 days after injection of cells or 50 mg/kg irinotecan 8, 11, 13, and 15 days after injection of cells. Reprinted with permission from ref 97. Copyright 2009 American Chemical Society.

demonstrate an interesting approach to the generation of dendrimers where cleavage of a single substrate by a tumor-specific enzyme leads to the release of multiple drug molecules, systems with an *in vivo* application have not been reported to date.

4.8. Complexation of Cisplatin to a Dendrimer. The clinical application of platinum-based chemotherapeutics is often hindered by their poor water solubility and high protein binding leading to systemic toxicity, particularly nephrotoxicity. Association of platinate drugs to dendritic drug carriers therefore has the potential to improve drug solubility, slow the excretion of the drug in urine (potentially limiting nephrotoxicity) and improve tumor biodistribution and therapeutic activity. However, there have been very few attempts to generate a platinum drug containing dendrimer, and of those that have been published only cisplatin has been associated with the dendrimer via complexation within the structure.

In one such study, Malik[100] and co-workers conjugated cisplatin to a G3.5 PAMAM dendrimer via complexation with a carboxylate linker. This improved the solubility of cisplatin and resulted in improved *in vivo* anticancer efficacy of the platinate against a platinate resistant tumor (B16F10), due to improved tumor targeting, internalization and retention of the drug. In addition, conjugation of cisplatin to the dendrimer increased the maximal tolerated dose of the nephrotoxic drug by 3- to 4-fold. In contrast, Bellis complexed cisplatin to the amine-terminated periphery of a PPI dendrimer, producing a system with overall poor aqueous solubility.[101] A more recent study complexed cisplatin via carboxylate terminal groups on a G1 and G2 triblock dendrimer consisting of citric acid–PEG–citric acid, with the idea that citric acid would provide a mechanism of uptake into highly metabolic tumor cells.[102] All dendrimers showed similar drug complexation levels of around 6% w/w, and release profiles showed a maximum of 5–8% platinum release at pH 5.4 following 72 h incubation. The G2-complexed drug also showed 2-fold greater *in vitro* cytotoxicity compared to free platinum against a partially resistant cell line, and approximately 8-fold greater cytotoxicity in several other cell lines.

5. SUMMARY OF THE ADVANTAGES AND DISADVANTAGES OF COVALENT AND NONCOVALENT DRUG ATTACHMENT

The hydrophobic interior scaffold of dendrimers combined with their multiple sites for hydrogen bonding and ionic interactions make them novel polymeric systems for encapsulating typically hydrophobic chemotherapy drugs and prolonging their circulation times. In addition, there is some evidence to suggest that encapsulation efficiency can be improved by PEGylating dendrimers, a modification that also further prolongs their plasma exposure and improves biocompatibility. In general, the advantages of noncovalent entrapment of chemotherapy drugs within the dendrimer scaffold include ease of drug loading, improved aqueous solubility of the drug and some benefit in terms of the therapeutic efficacy of the loaded drug for systems that retain drug for sufficiently long duration to allow EPR to improve passive drug delivery to the tumor (Table 2). In addition, this approach has the potential to reduce maximal plasma concentrations of cytotoxic drug, a factor that has often been linked to the severity of systemic toxicity.

Finally, some consideration needs to be given to the cost of synthesis and drug regulatory issues. Encapsulating chemotherapeutic drugs into the dendrimer scaffold has the advantage of reducing the number of steps taken to prepare the drug–dendrimer system in contrast to covalent drug association and therefore reducing the cost of synthesis. However, rapid clearance of the dendrimer via the urine, or rapid leakage of drug from the system, can negate any benefits achieved through reduced cost of synthesis, as higher doses need to be administered to achieve delivery of a sufficient proportion of drug. The converse of this drawback is that for applications where the EPR effect is not required, rapid release may be desirable, for example for rapid onset of therapeutic effect, or for simple solubilization enhancement applications. Encapsulation of drug may also avoid interaction between surface-bound drug and targeting groups in covalently conjugated systems. Additionally, noncovalently conjugated drug is not classified as a new drug entity, and therefore drug regulatory requirements for market approval are more relaxed than for covalently conjugated systems (keeping in mind that the dendrimer itself may be an NCE).

Despite these potential advantages, in general, the encapsulation approach has been met with problems associated with the burst release of many chemotherapeutics in plasma, often leading to little pharmacokinetic or therapeutic benefit compared with the administration of free drug (Table 2). For 5-fluorouracil and cisplatin, however, noncovalent entrapment and complexation have been the only approaches investigated for loading drug onto a dendrimer carrier. On the other hand, covalent association of chemotherapeutic drugs with dendrimers via surface conjugation through labile linkers significantly reduces initial burst release of drug and enables better control over the rate of drug release. The use of drug linkers that are specific to the microenvironment of the tumor or tumor-specific targeting ligands therefore allows the dendrimer carrier to accumulate in solid tumors with a high drug load to maximize exposure of cancer cells to the cytotoxic drug. This has the demonstrated benefit of reducing maximal plasma concentrations of free drug (reducing systemic toxicity) and maximizing the delivery of drug into solid tumors (improving chemotherapy compared to administration of free drug) (Table 2). The greatest downside to the conjugation approach, however, is the potential for drug liberation that is too slow to be effective *in vivo* or the release of less active forms of the drug (such as peptide modified MTX). Thus the search for increasingly sensitive and selective drug release triggers is probably worthy for both intra- and extracellular release. Furthermore, covalent association requires several additional synthetic steps, leading to greater heterogeneity of the final product and increased production costs. Ultimately, however, therapeutic benefit in terms of reduced tumor growth when compared to administration of free drug has more often been associated with covalent association of drug. In contrast, there have been relatively few *in vivo* studies that have demonstrated a significant improvement in chemotherapy or systemic toxicity when employing noncovalent drug association strategies.

6. CONCLUSION

The capacity of dendrimers to improve the solubility and biodistribution behavior of chemotherapeutics is evident from the literature, and is beginning to be translated into improved anticancer efficacy of a number of chemotherapeutic drugs. For those dendritic systems that have shown improved therapeutic efficacy or tumor distribution of chemotherapeutic drug in animal models of cancer, drug has generally been conjugated to the surface via tumor-labile linkers. Noncovalent drug encapsulation tends to result in rapid leakage of drug in biological fluids on account of reduced ionic interactions between drug and dendrimer and competition for drug binding by both soluble and tissue bound drug binding proteins. However, this is specific to both the encapsulated drug and the host dendrimer, and improved delivery of doxorubicin to tumor tissue in a noncovalent association complex has been observed relative to administration of free drug. The limiting interactions for noncovalent constructs can also be reduced by PEGylation or conjugation of surface targeting ligands, however in many cases, accumulation of encapsulated drug in tumors via the enhanced permeation and retention effect would be limited due to the typically extended periods of time required for efficient tumor accumulation. This can be overcome to some degree, however, via the use of cellular targeting ligands that can improve the rate and extent of drug delivery to tumor cells prior to clearance of the remaining drug delivery system.

Furthermore, leakage of encapsulated drug from dendrimer delivery systems is expected to reduce the benefit of association with the long circulating vector relative to surface conjugation. This is because of the increased exposure of noncancerous tissues to free drug and reduced accumulation of drug within tumors relative to more stable, surface conjugated systems. Consequently, it is apparent that the significant advantages of drug conjugation compared to drug encapsulation will more likely result in the ultimate development of therapeutic products from covalently bound drug–dendrimer conjugates than the use of dendrimers as solubilizing "unimolecular micelle" type approaches. It is also these authors' assertion that a focus of research effort on conjugated systems will therefore advance the dendrimer-based drug delivery field at a far greater rate, although investigators should ensure that constructs display

- linkers that display very rapid drug liberation in the tumor;
- linkers that allow liberation of ideally unmodified drug;
- long circulating characteristics as a minimum, but active targeting is also desirable;
- limited biodistribution in off-target organs;
- good aqueous solubility of the complex.

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Notes

The authors declare no competing financial interest.

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